Determining the effects of nutrition on the reproductive physiology of male mosquitoes

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University

by

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Abstract

The effects of nutrition on aspects of insect physiology such as body size, nutrition, and fecundity are well-studied in some species, but we lack a detailed picture of how nutrition influences the reproductive physiology of insects such as mosquitoes. Given that mosquitoes such as the Northern house mosquito, *Culex pipiens*, are vectors of many deadly diseases and their ability to quickly proliferate, understanding their reproductive physiology is critical. To uncover the relationship between nutrition and the reproductive physiology of mosquitoes, we reared larvae of *Cx. pipiens* on a standard lab diet and divided adult males among three different dietary treatments: low (3%), moderate (10%), and high (20%) sucrose. After one week, we measured male accessory gland (MAG) size and utilized nuclear magnetic resonance (NMR) spectroscopy to measure and identify metabolites within the MAGs. We also measured total lipid and protein content in the bodies of adult male mosquitoes. Finally, we allowed males to mate with females to determine whether male nutrition influenced female fecundity. We found that males raised on the 3% sucrose diet had significantly smaller MAGs compared to males from the 10% and 20% sucrose diets. We also found that diet affected the metabolic composition of the MAGs, where some metabolites became differentially abundant as a result of diet. We could not identify the differentially abundant metabolites, but we were able to identify a small number of metabolites present in all MAGs. Diet did not affect whole-body protein content, but surprisingly, lipid content was highest in males from the low dietary treatment. We did not observe a relationship between male diet and female fecundity. Taken together, our results demonstrate that adult male diet does affect their reproductive physiology.

1. Introduction

Insect physiology is responsive to multiple environmental factors that can vary throughout the lifetime of an organism. One factor that profoundly influences insect physiology is nutrition. Worker ants of *Azteca chartifex* (Hymenoptera: Formicidae) have higher thermal tolerances when fed carbohydrate-rich diets and are less tolerant to changes in temperature when only water was available (Bujan and Kaspari 2017). Nymphs of the grasshopper *Schistocerca americana* (Orthoptera: Acrididae) emerged as smaller adults and had reduced lipid stores when fed lower nutrition diets, while nymphs that were raised on higher nutrition diets emerged with larger body sizes and with greater lipid stores (Hahn 2005).

Aside from general impacts on insect physiology, nutrition affects fecundity in a variety of insects. Two species of hyperparasitoid wasps, *Gelis agilis* (Hymenoptera: Ichneumonidae) and *Lysibia nana* (Hymenoptera: Ichneumonidae), had significantly different reproductive success when fed diets consisting of different sugar types such as glucose, melibiose, maltose, trehalose, and mannose (Harvey et al. 2012). Female mosquitoes of *Ochlerotatus atropalpus* (Diptera: Culicidae) produced fewer eggs when undernourished as larvae (Telang and Wells 2004), while adult female mosquitoes of *Aedes aegypti* (Diptera: Culicidae) produced more eggs when fed weekly blood meals versus females that were fed either none or only one blood meal (Joy et al. 2010). The implications of nutrition affecting fecundity in mosquitoes are important, as mosquitoes can produce hundreds of offspring in their lifetimes. A single female of *Culex pipiens* (Diptera: Culicidae) can produce ~500-1,000 total eggs across multiple egg rafts (Madder et al. 1983). Given that mosquitoes are prolific vectors of deadly diseases (WHO 2019), and the capacity of *Cx. pipiens* specifically to transmit multiple diseases such as West Nile virus and St. Louis encephalitis (Farajollahi et al. 2011), this rapid proliferation of mosquitoes poses a

serious threat to public health. In 2018, 2,647 cases of West Nile virus were reported in the United States (CDC). Understanding how nutrition affects the fecundity of mosquitoes is critical to understanding the reproductive physiology of these deadly disease vectors.

Male physiology can also impact female reproductive behavior and fecundity. Male accessory glands (MAGs) are a critical reproductive organ in several insects, as they have been shown to play multiple roles in insect reproduction. Products of the MAGs, such as seminal fluid proteins, in *Drosophila melanogaster* (Diptera: Drosophilidae) stimulate egg production in females (Herndon and Wolfner 1995). Female refractoriness to re-mating decreased in *Ceratitis capitata* (Diptera: Tephritidae), in which MAG products likely played a role (Miyatake et al. 1999). MAGs have been shown to possess similar functions in mosquitoes. Helinski et al. (2012) demonstrated that females of *Ae. aegypti* and *Ae. albopictus* were less likely to re-mate upon being injected with seminal fluid proteins alone. Females of *An. gambiae* also exhibited decreased receptivity to re-mating after mating with spermless males, suggesting that products in the MAGs alone are influencing post-mating behaviors in female mosquitoes (Thailayil et al. 2011). Additionally, seminal fluids produced in the MAGs can induce female mosquitoes to take a blood meal and digest that blood meal quickly, to increase oogenesis and oviposition, and induce immune responses (see reviews by Baldini et al. 2012; Meuti and Short 2019).

Male nutrition also influences male reproductive behavior as well as the factors that MAGs produce. Males of the Mediterranean fruit fly, *C. capitata*, that were fed high-protein diets were more likely to mate than their protein-deprived counterparts, and females that mated with protein-fed males were less likely to re-mate afterwards (Blay and Yuval 1997). Males of *D. melanogaster* that were fed higher nutrition diets fathered more offspring compared to males raised on low nutrition diets (Fricke et al. 2008). Nutritional differences in male mosquitoes have

also been observed to affect female fecundity. Like fruit flies, males of *Ae. aegypti* that consumed a richer diet fathered more offspring than males raised on a low-nutrition diet (Clifton et al. 2014). The precise mechanisms underlying these differences are still unclear, but it is likely that differences in nutrition among male mosquitoes affect components of the ejaculate.

As the exact underpinnings of nutrition and MAG products have not been well-characterized in many species, metabolomics could be useful in investigating these unknown areas. Metabolomics using Nuclear Magnetic Resonance spectroscopy (NMR) has been used to study and characterize a variety of reproductive tissues and products in several mammals such as ungulates, rats, and humans (Gérard et al. 2015; Banerjee et al. 2012; Mumcu et al. 2020). All three of these studies identified several metabolites in the reproductive organs of all mammalian species, such as glucose, lactate, citrate, and multiple amino acids. For more general information about NMR methods used in these papers, see the review by Wishart (2008).

Metabolomic studies of reproductive organs, and more specifically MAGs, have been conducted in some insect species, but this is still a growing area of research (see review by Snart et al. 2015). A study of the transcriptome, proteome, and metabolome of the spermatophore of the firefly, *Photinus pyralis* (Coleoptera: Lampyridae), characterized the composition of these nuptial gifts that males provide to females (Al-Wathiqui et al. 2016). This study identified a few small-molecule compounds associated with firefly defense that were produced within MAGs. Many other small molecules were measured in the glands but could not be identified using existing database information. Additional studies have characterized some metabolites in the reproductive tract of some dipteran species. Chintapalli et al. (2013) identified a small number of polar metabolites in the MAGs of *D. melanogaster*, including n-acetylhistamine, s-inosylmethionine, and decarboxy S-adenosylmethionine, along with metabolites for many other

tissues including the testes. Pondeville et al. (2008) found that males of *Anopheles gambiae* (Diptera: Culicidae) produce and store 20-hydroxyecdysone (20E), a steroid hormone important to vitellogenesis in females, in the MAGs. Juvenile hormone (JH), a molecule involved in stimulating egg follicle development in females, is produced in the MAGs of *Ae. aegypti* (Clifton et al. 2014).

Multiple studies demonstrate that female mosquito fecundity increases in larvae of *O. atropalpus* and adults of *Ae. aegypti* when females have access to high-nutrition diets (Telang and Wells 2004; Joy et al. 2010), but none of the mosquito genera used in these studies included *Culex*. Does a similar effect occur if males of *Culex pipiens* are raised on diets of different nutrition levels? Similarly, we know that poor nutrition reduces overall body size (Hahn 2005), but whether this effect on body size is translated into changes in MAG size is unknown. Furthermore, it is not clear whether nutrition has any effect the composition of metabolites within the MAGs. This study aimed to improve our understanding of how nutrition affects aspects of reproductive physiology in mosquitoes of *Cx. pipiens*, including how MAG composition responds to changes in dietary quality.

2. Methods

2.1 Mosquito rearing

All *Cx. pipiens* mosquito larvae (Buckeye strain) used in the experiments were reared on the same standardized diet of ground Tetramin fish flakes, and larval and adult mosquitoes were reared in an environmental chamber set to 27°C and 80% RH with 14 hours of light and 10 hours of darkness. Upon pupation, mosquitoes were separated by sex. Male pupae were then randomly divided into three groups of equal numbers (n = 50-150 males/treatment) and placed into cages. Each cage of adult male mosquitoes was given access to RO water and either a 3%, 10%, or 20%

sucrose solution. Each cage of males was allowed to feed on their respective diets *ad libitum* for seven days.

2.2 Effects of diet on male accessory gland size

Male accessory gland (MAG) size was measured using a dissecting microscope (Leica, 50X magnification). The accessory glands from 20, 1-week-old males from each treatment were removed via dissection. MAG area was estimated by taking length and width measurements of each gland using the Leica Application Suite V4.12 software and calculated using the equation for the area of an ellipse ($A = ½w * ½l * \pi$).

2.3 Effects of diet on the metabolic composition of male accessory glands

Metabolic content was measured using nuclear magnetic resonance (NMR) spectroscopy. Metabolites were extracted from MAGs using two modified versions of a previously established procedure (Wu et al. 2008) allowing us to measure differences in aqueous and lipid-based metabolites. To measure differences in water-soluble metabolites, the accessory glands of 10 males/sample were dissected (n = 5 pooled biological replicates from each treatment) and frozen at -20°C in 50 µl DI water in microcentrifuge tubes. To extract metabolites, the samples were thawed and re-frozen three times before being mixed with 400 µl cold (200 proof) EtOH and 150 µl DI water. Each sample was sonicated for 30 min and then vortexed for 60 s. The samples were frozen again at -20ºC for 1 hr before being removed again. All samples were then centrifuged for 10 min at 14,000 rcf at 4ºC. Supernatant from each sample was collected into clean glass vials and were then completely dried in a CombiDancer evaporator (Hettich, Tuttlingen, Germany) using a custom program. To prepare the dry samples for NMR measurements, 600 µl D_2O and the reference substance, deuterated trimethylsilylpropanoic acid (TSP) in 50 µl D_2O, were added

to each vial. The pH of each sample was then adjusted to fall between 7.35-7.44 before being transferred to glass NMR tubes. Additionally, once the MAG EtOH extract samples were measured individually, they were pooled together, evaporated, dissolved in D2O, pH-adjusted, and measured again in order to obtain a more detailed 2D spectrum representing the general metabolites found in all the MAGs. This pooled spectrum was used to identify unknown signals, utilizing metabolite NMR spectra databases to identify metabolites in our MAG samples.

To measure differences in lipid-soluble metabolites, the accessory glands from a single male mosquito were dissected (n = 10 biological replicates/treatment) and added to 10 µl DI water in a microcentrifuge tube. All samples were frozen at -20ºC and thawed three times. Following this, 80 µl H_2O and 400 µl MeOH were added to each sample. All tubes were sonicated for 30 min before adding 400 µl $CHCl_3$ and 200 µl H_2O to each sample. Each sample was frozen at -20ºC for 1 hr, after which all were centrifuged for 10 min at 14,000 rcf and 4ºC. The centrifugation process caused the contents of each tube to separate into distinct aqueous layers of MeOH and $CHCl_3$, whereby each layer was pipetted into individual tubes. All chloroform-extracted samples were evaporated and then supplemented with 650 µl $CDCl_3$ containing octamethylcyclotetrasiloxanebefore (OMS) as a reference substance, and then transferred to glass NMR tubes for measurement. All samples from both extraction procedures were measured using a Bruker Avance III HD Ascend 850 MHz spectrometer equipped with a cryogenic probe (Bruker, Billarica, MA), located in the Chemical and Biomolecular Engineering and Chemistry Building on Ohio State University's campus. 1D 1H NOESY spectra were recorded for all samples. Samples in deuterium oxide were recorded using pre-saturation for residual water suppression. 2D 1H-13C HSQC spectra were recorded for all chloroform spectra,

and for one pooled sample in D_2O. Spectral data for both procedures was pre-processed using TopSpin 3.6.1 (Bruker).

2.4 Effects of diet on lipid and protein content

To measure macromolecular content, male mosquitoes were killed via freezing after the seven-day feeding period. Lipid content was assessed using a vanillin phosphoric acid lipid assay initially developed by Van Handel (1985) but modified to allow for mosquito lipid content to be measured using a microplate reader (Meuti et al. 2015). In brief, the wet mass of eight males was recorded, and lipid was extracted using chloroform. After measuring the lipid concentration in each mosquito according to Van Handel (1985) and Meuti et al. (2015), the values were standardized by dividing concentration by the mass of each respective mosquito. Protein content in 8 individual mosquitoes was assessed by using a Bradford protein assay (Bio-Rad) and again total protein was divided by fresh mosquito weight.

2.5 Assessing the impact of male diet on female fecundity

To determine whether the diet male mosquitoes consumed influenced female fecundity, 50 one-week old adult females that had been raised on a 3% diet were placed into cages containing 150 male mosquitoes that had consumed either a 3%, 10% or 20% sucrose diet. Sucrose solutions were removed from each cage right before males and females were combined. Females were allowed to feed on chicken blood using an artificial blood-feeding system (Hemotek, UK) two to three days following their introduction to male cages. Following blood-feeding, oviposition water was placed in each cage so that females could lay egg rafts. The total number of egg rafts laid within each cage, as well as the number of eggs within each raft and the total number of larvae that hatched from each raft were recorded.

2.6 Data Analysis

All data analysis for MAG size, whole-body lipid and protein levels, and fecundity was performed in JMP 14. One-way ANOVA and post hoc Tukey-HSD tests were used for the MAG size data and the whole-body lipid and protein data. The fecundity data were analyzed using Kruskal-Wallis H tests. NMR spectra were processed in TopSpin 3.6.1. NMR data were imported into R 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) using the *mrbin* package (*v1.1*; Klein 2020). Spectra were scaled to the TSP or OMS signals and then split up into equally sized bins. For the EtOH extract data, bin widths were set at 0.003. The signal-to-noise ratio was set to 10 and the noise threshold was set to 0.75, and noise was then removed from all bins. For the chloroform extracts, bin widths were set to 0.02. The signal-to-noise ratio and noise threshold were set to 30 and 0.75, respectively. Noise was removed from the binned data. Spectra were scaled to the TSP or OMS signal for the EtOH and $CHCl_3$ extracts, respectively. All figures were constructed in R 3.6.1. PCA and Spearman's correlation tests were performed on the binned NMR data in R. P-values were corrected for False Discovery Rate controlling at a level of 0.2.

3. Results

3.1 Effects of diet on male accessory gland size

The concentration of sucrose in the diet significantly affected male accessory gland size (Figure 1; One-way ANOVA; $F_{2,116;\,0.05} = 4.65$, $p = 0.0115$). Specifically, male mosquitoes that consumed a 3% sucrose solution had significantly smaller accessory glands than males from both the 10% treatment ($p = 0.0334$) and the 20% treatment ($p = 0.0191$). The average accessory gland size of males from the 3% diet treatment was 0.0137 ± 0.0022 mm^2, and the average gland

sizes of the males from the 10% and 20% treatments were 0.0149 ± 0.0018 and 0.0150 ± 0.0022 mm^2, respectively. Male accessory glands were not significantly different between males that consumed the 10% and 20% sucrose diets (p = 0.976).

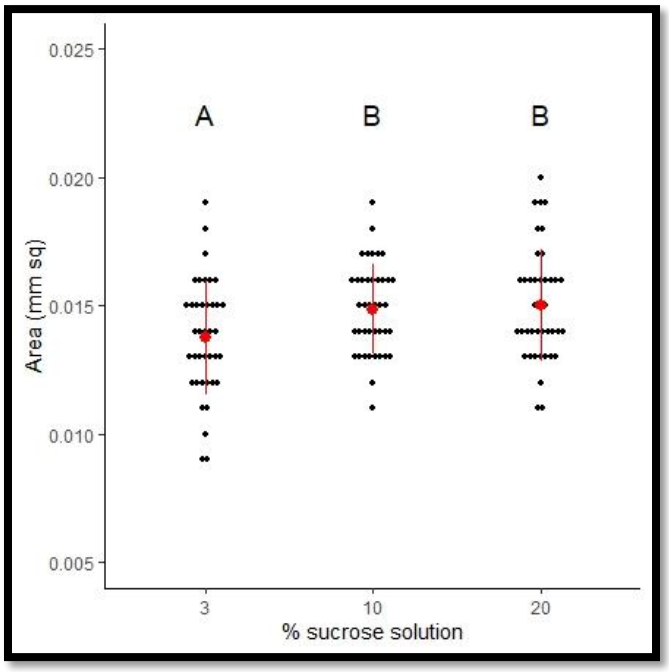


Figure 1: Nutrition affects MAG size. Males from the 3% treatment had significantly smaller accessory glands than males from both the 10% and the 20% treatments (One-way ANOVA and Tukey-HSD post-hoc tests; $F_{2,116;\,0.05}$ = 4.65, p = 0.0115). Different letters indicate significant differences. Black dots represent individual glands, red dots and bars represent sample means and standard deviations, respectively.

3.2 Effects of nutrition on the metabolic composition of male accessory glands

The untargeted approach, where a pooled sample of all EtOH extracts from MAGs from all 3 dietary treatments yielded a small number of identifiable metabolites. The Human Metabolome Database (Wishart et al. 2018), or HMDB, was used as a reference for comparing peaks from our 2D MAG spectra to existing spectra. Using this database, we were able to identify three different molecules: glycerol, lactic acid, and maltotetraose (Fig. 2a). Interestingly, the pooled spectrum showed that glycerol was present in the MAGs in much higher

concentrations than any other metabolite (Fig. 2b).

a)

b)

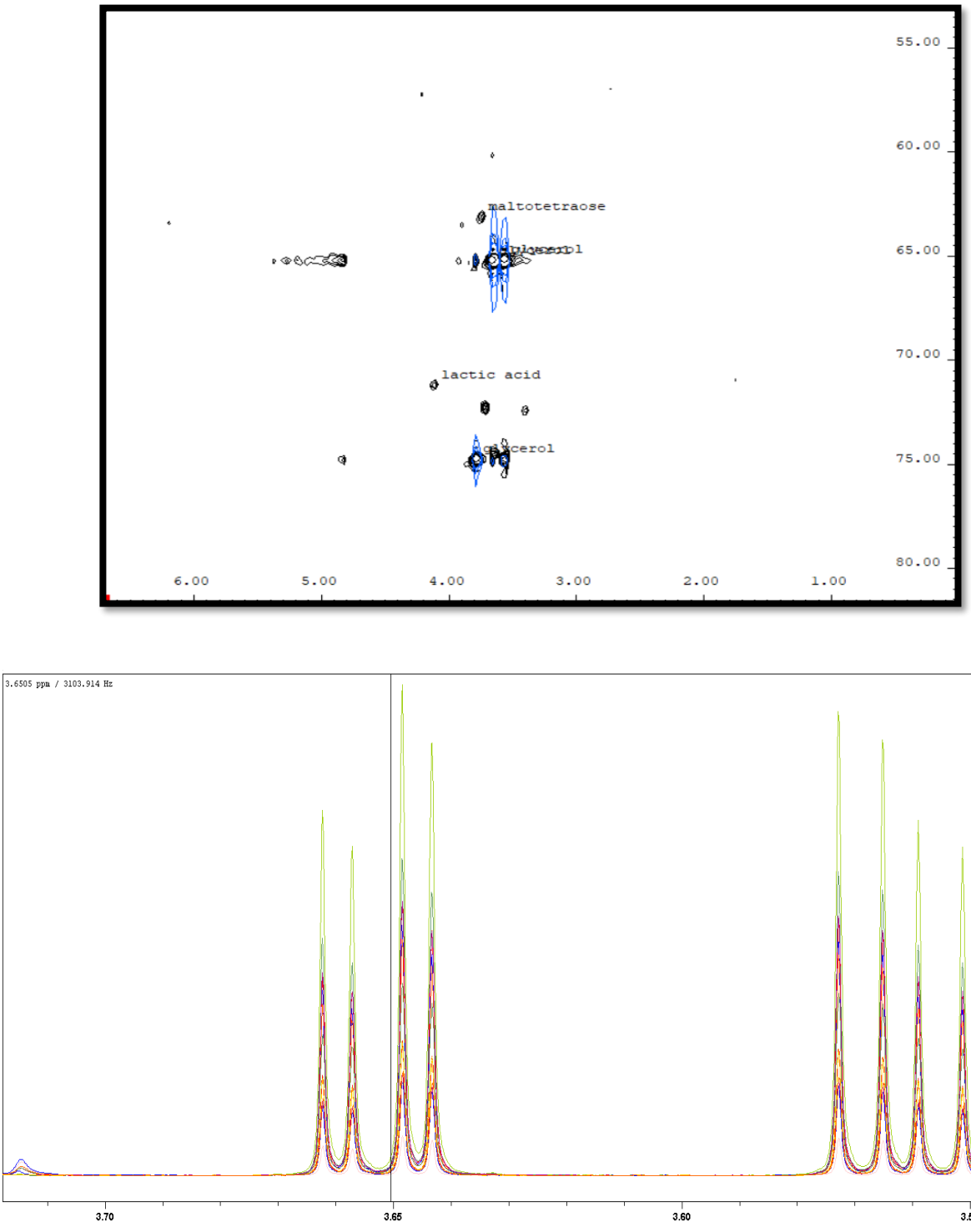


Figure 2: Portion of 2D NMR spectrum of pooled MAG EtOH extracts showing identified molecules (a) and a portion of the 1D NMR spectrum of pooled MAG EtOH extracts, where the large peaks seen represent the molecule glycerol (b).

Initially, PCA of the MAG EtOH extracts revealed that there is not an obviously strong correlation between metabolite measurements and dietary treatment (Fig. 3).

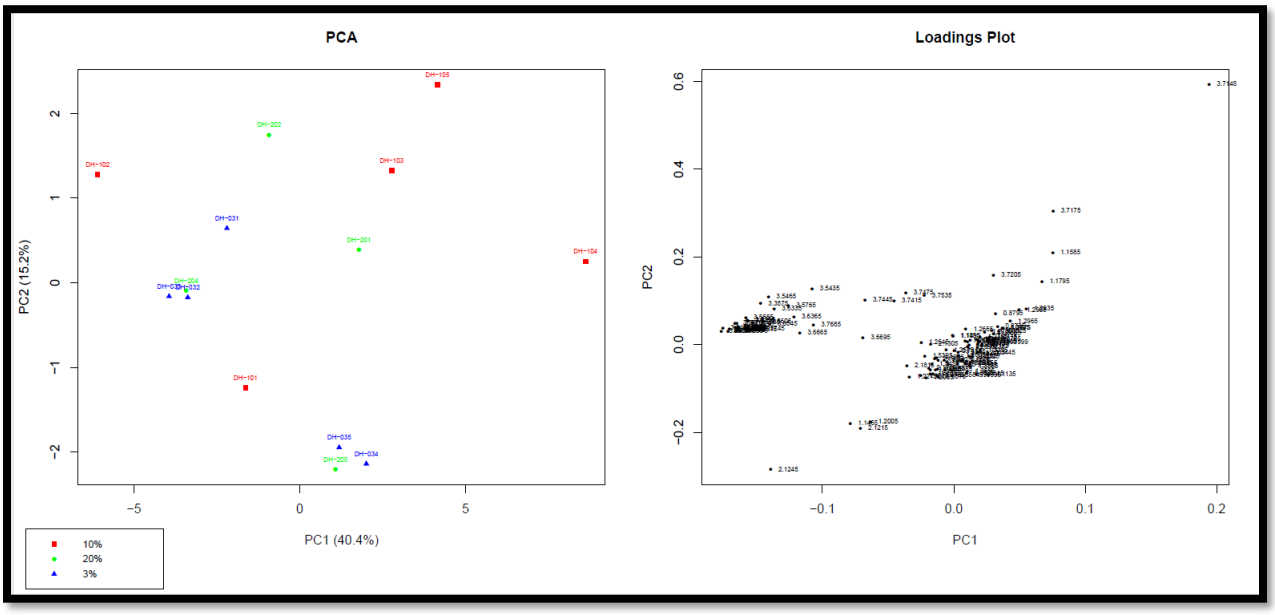


Figure 3: PCA plot and loadings plot of MAG EtOH extract data. No strong correlation was observed between metabolite measurements and sucrose concentration.

Spearman's correlation was then used to analyze the binned signals for correlation to sucrose content. A histogram of raw p-values indicates true significant correlations, as evidenced by an enrichment of p-values close to 0 (Figure 4). After controlling for multiple testing, it was found that there were 26 bins (out of 125 total) that were significantly correlated with dietary sucrose content (Fig. 5; Table 1). Unfortunately, none of the peaks that fell within the significantly correlated bins could be initially identified using information in the HMDB.

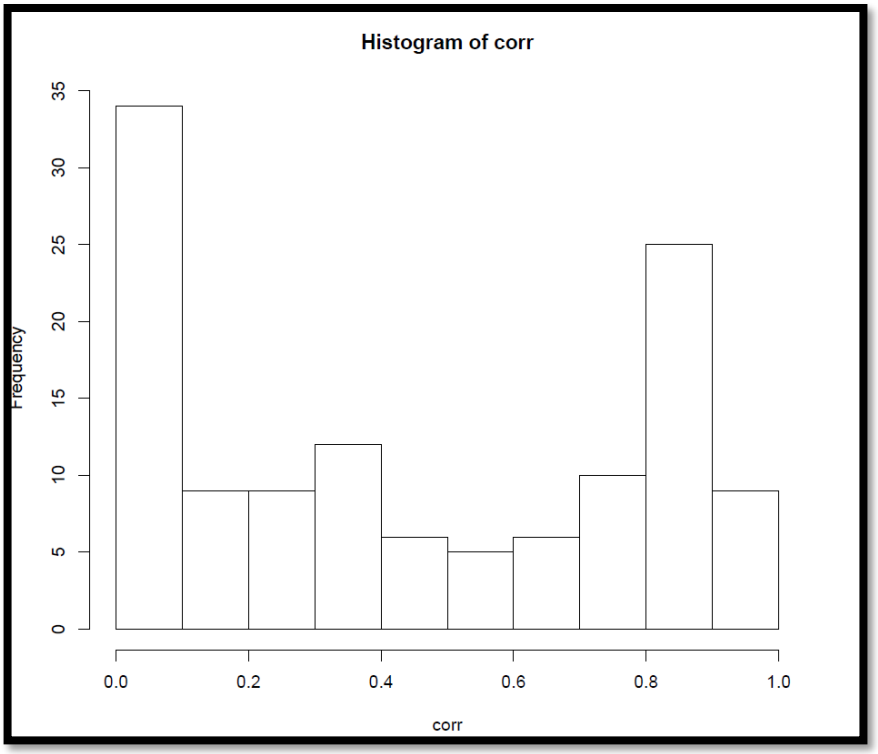


Figure 4: Histogram of correlation for MAG EtOH extracts (p-values are on the x-axis).The large bin near 0.0 indicates that several metabolites were significantly affected by dietary treatment.

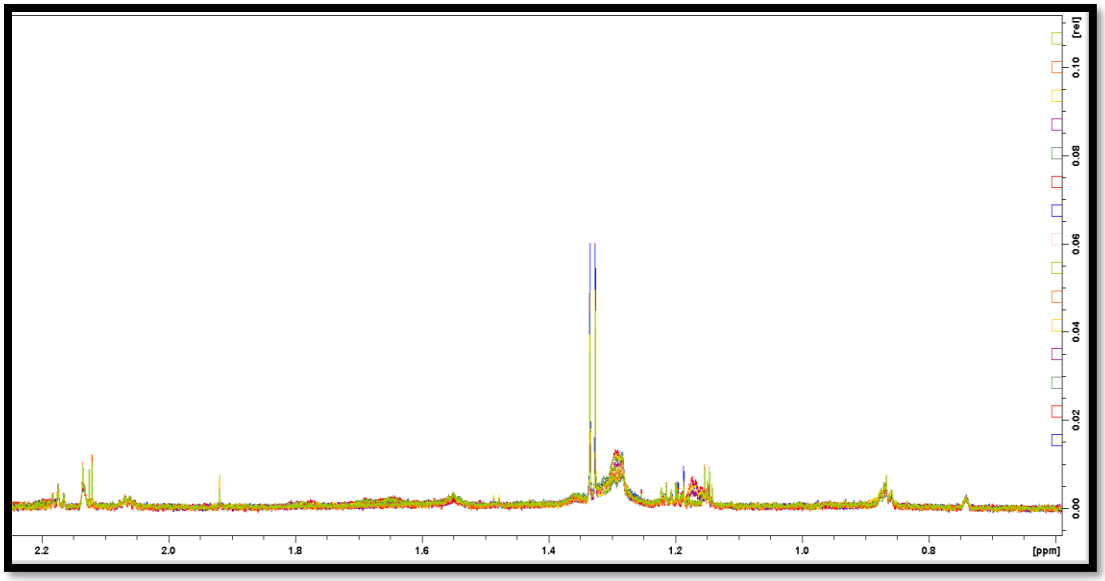


Figure 5: NMR spectra from all MAG EtOH extract samples containing range of significant bins. Each colored line represents a different sample.

0.8615	0.8675	0.8705	0.8735	0.8765	0.8795	1.1945
1.2815	1.2845	1.2875	1.2905	1.2935	1.2965	1.2995
1.3025	1.3055	1.3085	1.3115	1.3145	1.3205	1.3535
1.5485	2.0615	2.1215	2.1245	2.1335		

Table 1: Positions of the significantly correlated bins from the MAG EtOH extractions (1H ppm values).

For the MAG chloroform extracts, PCA did not reveal a strong correlation between dietary treatments (Fig. 6). While the histogram of p-values again suggests that the concentration of sucrose in the diet affected the abundance of several metabolites (Fig. 7), after correction for multiple testing only one (3.155 ppm) out of 206 total bins was found to be significantly correlated with dietary treatment (Fig. 8). The peak for this bin could not be identified using information from HMDB.

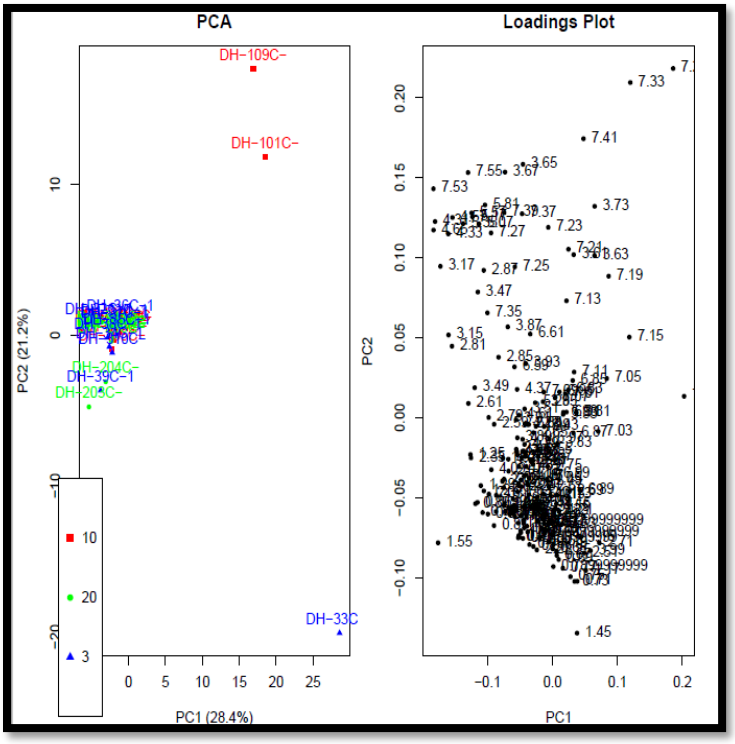


Figure 6: MAG chloroform extract PCA and loading plot. No strong correlation was observed between metabolic measurements and sucrose concentration.

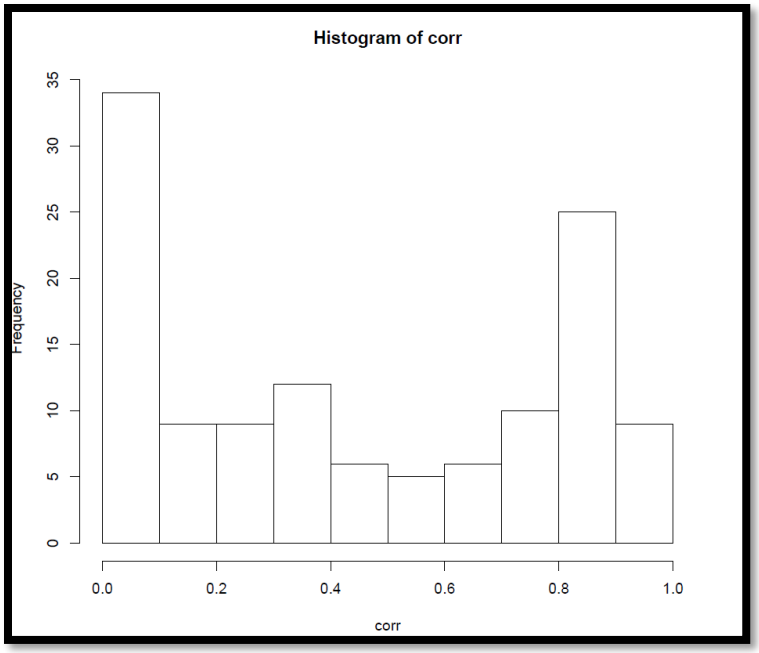


Figure 7: Histogram of correlation for MAG chloroform extracts (p-values on the x-axis).

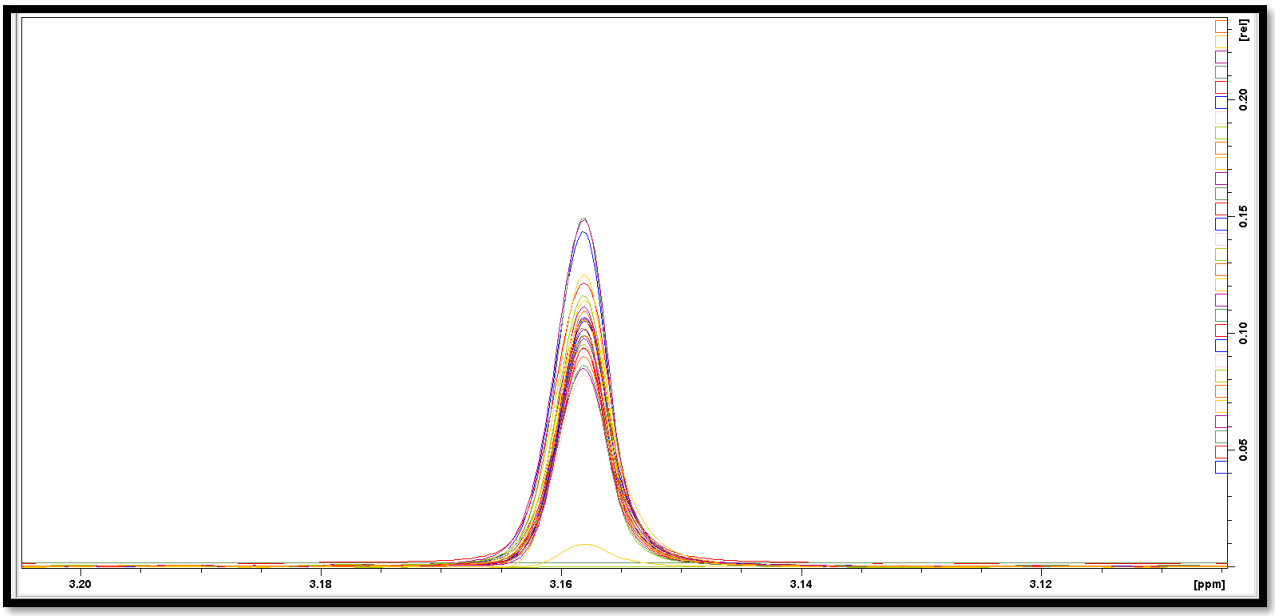
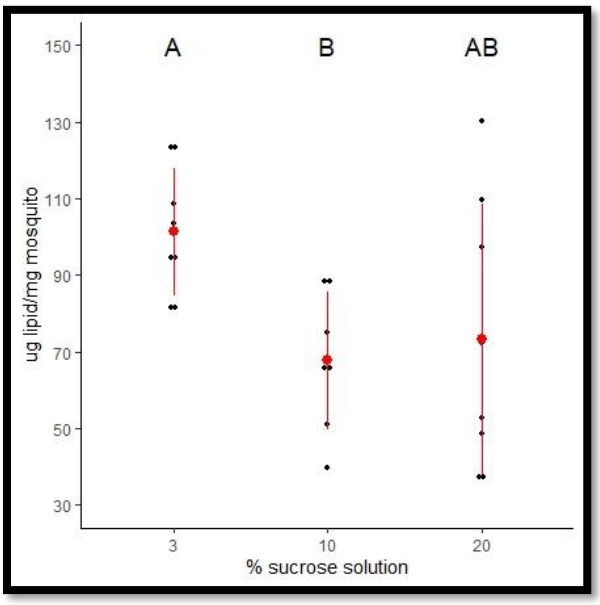


Figure 8: NMR spectra from all chloroform MAG extracts containing the peak from the single significant bin. Each colored line represents a different sample.

3.3 Effects of diet on lipid and protein content

Diet significantly impacted lipid content (One-way ANOVA; $F_{2,20;\,0.05} = 3.98$, $p = 0.0350$). Surprisingly though, males that consumed the 3% sucrose diet had 1.3 fold more lipid content (101.3 ± 16.6 µg lipid/mg mosquito) than males from the 10% treatment (67.7 ± 18.1 µg lipid/mg mosquito), and this difference was significant ($p = 0.0440$). However, the lipid content between males in the 3% and 20% diet (73.3 ± 35.3 µg lipid/mg mosquito) were not significantly different ($p = 0.0893$, Fig. 9a). The concentration of sucrose did not affect the protein content of the males (Fig. 9b; One-way ANOVA; $F_{2,\,20;\,0.05} = 0.099$, $p = 0.99$). Males that consumed 3%, 10% and 20% diets had respective average protein contents of 56.9 ± 22.2, 58.3 ± 22.8, and 57.5 ± 14.7 µg protein/mg mosquito.

a)



b)

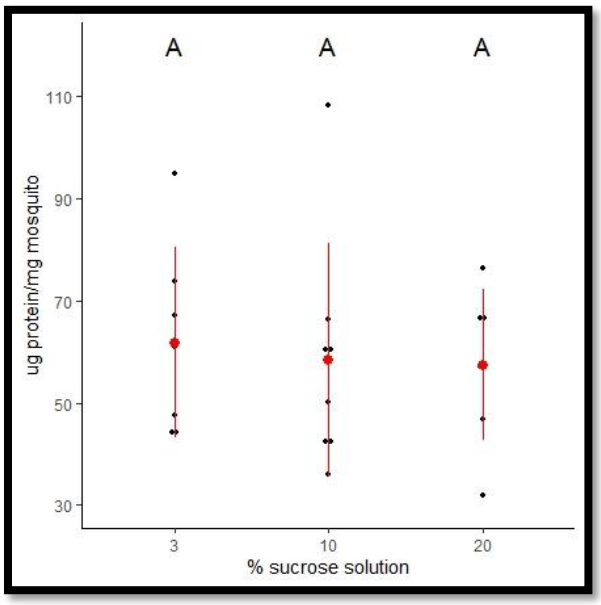


Figure 9: Dietary treatment affected whole body lipid content (a) but not protein (b) in male mosquitoes. Lipid content in the 3% treatment males was significantly higher than that of the 10% treatment males (One-way ANOVA and Tukey HSD post-hoc tests; p = 0.044). No significant differences were found in total protein content between males from each treatment (One-way ANOVA and Tukey HSD post-hoc tests; p = 0.9902) Black dots indicate the data from individual male mosquitoes, while red dots and lines indicate the mean and standard deviation. Different letters indicate significant differences among dietary treatments.

3.4 Assessing the impact of male diet on female fecundity

The diet of adult male mosquitoes did not cause any significant differences in female reproductive success. Females that mated with males that consumed 3% sucrose diet produced 17 egg rafts, while females that mated with males that consumed 10% sucrose produced 19 egg rafts, and females that mated with males that consumed 20% sucrose produced 12 egg rafts. The average number of eggs per raft in the 3%, 10%, 20% treatments was 171 ± 82, 152 ± 93, and 180 ± 82, respectively. Notably, females that mated with males that had consumed 3% sucrose showed the greatest variation in the number of eggs/raft ranging from 46 to 299 eggs/raft. The egg raft sizes of females that had mated with males that consumed 10% sucrose ranged between 34 and 369. The smallest and largest egg rafts laid by females that had mated with males from the 20% treatment contained 52 and 337 eggs, respectively. Given the high degree of variation between the number of eggs/raft, no significant differences were found between the average number of eggs in each raft between treatments (Fig. 10a; Kruskal-Wallis H test; $\chi^2_{2;\,0.05} = 0.863$; $p = 0.650$).

The number of larvae that hatched from each egg raft also did not differ significantly between treatments (Fig. 10b; Kruskal-Wallis H test; $\chi^2_{2;\,0.05} = 0.645$; $p = 0.724$). The mean number of larvae that hatched in the 3%, 10%, and 20% treatments was 74 ± 61, 67 ± 72, and 82 ± 64 larvae, respectively. Each treatment had one egg raft that did not produce any larvae, and there were multiple egg rafts in each treatment that produced < 10 larvae (Fig 10b). The mean hatch rate, defined as the number of larvae hatched divided by the number of eggs in the corresponding raft, was highest in the 20% treatment at 0.46 ± 0.25, and the 3% and 10% treatments had respective hatch rates of 0.39 ± 0.25 and 0.35 ± 0.27. However, the hatch rate did

not differ significantly between treatments (Fig. 10c; Kruskal-Wallis H test; $\chi^2_{2;\,0.05} = 1.52$; p = 0.468).

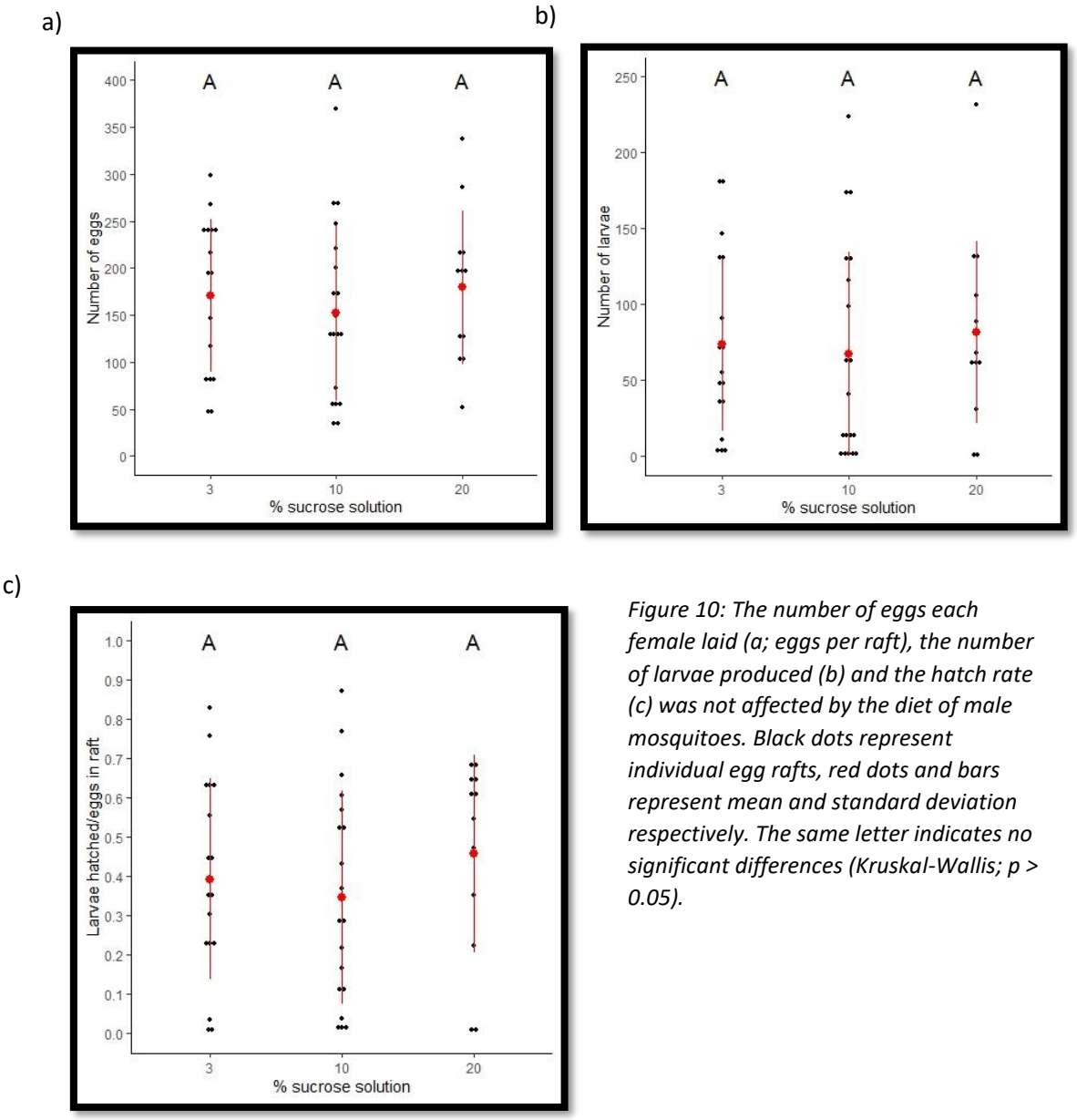


Figure 10: The number of eggs each female laid (a; eggs per raft), the number of larvae produced (b) and the hatch rate (c) was not affected by the diet of male mosquitoes. Black dots represent individual egg rafts, red dots and bars represent mean and standard deviation respectively. The same letter indicates no significant differences (Kruskal-Wallis; p > 0.05).

4. Discussion

Our results demonstrate that nutrition affects the size of the MAGs in males of *Cx. pipiens*. Specifically, males raised on a low-nutrition diet had significantly smaller MAGs in comparison to males raised on moderate and high-nutrition diets (Fig. 1). Analysis of NMR spectra taken from the EtOH-based MAG extraction revealed that there were some significant

differences in MAG composition between treatments, but we could not determine which specific molecules differed. However, we were able to identify a small number of small metabolites, including glycerol, lactic acid, and maltotetraose, that were present in all MAGs. Whole-body macromolecule analysis showed that males from the 3% sucrose solution treatment had significantly higher levels of lipids in their bodies when compared to males from the 10% treatment, but protein levels did not differ between treatments. Finally, the amount of sucrose in the male diet did not affect female fecundity.

Through our investigation, we discovered that adult male mosquitoes raised on low-nutrition diets for seven days had smaller MAGs when compared to males from moderate and high-nutrition diets. The influence of nutrition on overall body size in insects has been reported by several researchers, who report that nutrition affects molecules, such as JH and ecdysteroids, that are involved in multiple developmental pathways (reviewed by Nijhout 2003; Koyama and Mirth 2018). Much attention has been given to overall development and body size in insects, but there is less information regarding the relationship between nutrition and the size of reproductive organs. Arnqvist and Thornhill (1998) found that nutritional stress reduced genital size in males of *Gerris incognitus* (Hemiptera: Gerridae), but other studies (Tang et al. 2011) have found that male insect genital size is subject to little change when nutritionally restricted. Our work here represents the first time that nutrition has been shown to impact the size of the male accessory glands in any insect.

NMR spectroscopy allowed us to identify some small metabolites in the MAGs of our mosquitoes. The pooled sample of MAGs extracted via ethanol contained glycerol, lactic acid, and maltotetraose, along with several other unidentifiable molecules. Interestingly, the spectrum produced from the pooled sample showed that glycerol was much more abundant in the MAGs

than other molecules (Fig. 2b). Chintapalli et al. (2013) were able to identify a small number of MAG metabolites in *D. melanogaster* using Hydrophilic Interaction Liquid Chromatography (HPLC), but none of the metabolites they identified were present in MAGs from males of *Cx. pipiens*. Al-Wathiqui et al. (2016) also identified small molecules within *P. pyralis* spermatophores using mass-spectrometry, but only a small number of them could be successfully identified. We believe that our work represents the first step towards using NMR methods to identify MAG metabolites in insect tissues. In the future, these findings can be built upon by including larger amounts of tissues per sample which could provide more detailed spectra that may lead to the identification of additional metabolites. Additionally, our work shows that it is feasible to use NMR spectroscopy to identify metabolites in small amounts of organs and tissues in insects.

Our NMR spectra also show that differences in nutrition affect MAG composition in *Cx. pipiens*. However, we were not able to specifically identify the molecules that became differentially abundant, possibly because several of the different signals may stem from macromolecules, which are difficult to identify using NMR spectroscopy. To our knowledge, this is the first time that the effects of nutrition on MAG metabolites has been studied in any insect. Previous studies have shown that differences in nutrition do lead to differences in insect body size (Hahn 2005) and reproductive success (Harvey et al. 2012, Telang and Wells 2004; Joy et al. 2010; Fricke et al. 2010), but these studies did not focus on how nutrition affected the metabolic composition of insect organs and tissues. Future studies could utilize different spectroscopy methods such as liquid chromatography-mass spectrometry (LC-MS) to identify larger molecules, such as proteins, that become differentially abundant in response to dietary quality.

Despite the likely differences in specific proteins in the MAGs, the diet of adult male mosquitoes did not appear to significantly affect whole-body protein levels. However, a lipid assay revealed that males that had consumed a 3% sucrose diet had significantly more whole-body lipid content than males that had consumed a 10% diet. Hahn (2005) found that nymphal grasshoppers of *S. americana* that consumed low-nutrition diets had significantly less lipid stores in adulthood when compared to grasshoppers that were raised on an intermediate diet and, similar to our results, protein content in adults was not significantly affected by dietary quality. Moths of *Heliothis virescens* (Lepidoptera: Noctuidae) that were raised on higher-protein diets had higher stores of protein later in their lives (Telang et al. 2002). Telang and Wells (2004) showed that feeding mosquito larvae of *O. atropalpus* rich and intermediate diets caused adults to have higher lipid and protein content relative to mosquitoes that were fed poor diets as larvae. These previously published results are inconsistent with our finding that mosquito adults from the poor dietary treatment had the greatest lipid stores in their bodies. Notably, Telang and Wells (2004) utilized a similar, but somewhat modified, version of the Vanillin lipid assay that we used (Van Handel 1985). A key difference in the two studies is that Telang and Wells (2004) varied the diets of mosquito larvae, while our study was designed with a standard larval diet and different adult diets. It is also possible that our results were in part caused by males in each cage having access to their assigned sucrose solution diets right up until their euthanization. If males from the 3% treatment had more undigested sucrose in their bodies than males from the other treatments, then it is possible that the Vanillin reagent reacted with the extra sugar and made it appear that those males had more fat. Our results, taken into consideration with previous results from other studies, warrant further investigation into how altering the diets of adult mosquitoes

could affect whole-body lipid concentration. Repeating the lipid assay described in this study could help clarify the result that we observed.

We did not observe any significant differences in reproductive success between male mosquitoes from different dietary treatments (Fig. 10). These results differ from previous studies that have examined the relationship between nutrition and fecundity. The effects of female insect nutrition on fecundity is well-documented. Access to richer nutrition sources in females of hyperparasitoid wasps, as well as multiple mosquito species, resulted in increased reproductive success (Harvey et al. 2012; Telang and Wells 2004; Joy et al. 2010). Male diet has also been shown to be positively correlated with fecundity, as seen in both *D. melanogaster* and *Ae. aegypti* (Fricke et al. 2010; Clifton et al. 2014). Clifton et al. (2014) used a somewhat similar experimental setup to ours where sucrose solutions were made available to male mosquitoes. However, our study added a 10% sucrose solution alongside 3% and 20% solutions. Additionally, the fecundity portion of our study was set up so that there was a 3:1 ratio of males to females. Clifton et al. (2014) utilized a 1:1 ratio of males to females. Does the ratio of males to females in a cage influence the results of fecundity experiments? This question could be answered by performing additional fecundity experiments using males raised on varied diets, where multiple male:female ratios are used. Another avenue of investigation that emerges from this study is the possibility of evaluating whether naturally occurring sugars instead of sucrose, such as fructose, glucose, or xylose affect male and female mosquito fecundity. Harvey et al. (2012) fed wasps of *L. nana* and *G. agilis* different sugars as well as honey and observed that wasps raised on mannose had significantly lower reproductive success compared to wasps raised on glucose, melibiose, maltose, or trehalose.

Our results show that nutrition plays an important role in the reproductive physiology of male *Cx. pipiens*. Adult males that were reared on nutritionally deficient diets had significantly smaller accessory glands. Additionally, we were able to use NMR spectroscopy to measure metabolites in small amounts of MAG tissues for the first time in any insect. We found that some molecules in the MAGs became differentially abundant as a result of dietary quality. This study is among the first to evaluate the effects of nutrition on large-scale differences MAG composition in insects. While the identification of all metabolites in the MAGs proved to be difficult, we were able to identify a small number of critical metabolites. In doing so, we discovered that glycerol is much more abundant than any other molecule present in the MAGs. With further refinement, these methods can be utilized to further characterize the metabolome of mosquito MAGs as well as a variety of other insect organs and tissues. Doing so will allow us to better understand the metabolic composition of important reproductive organs in mosquitoes as well as other insects. Future work should expand on our results by varying the diets of both larvae and adults in other insect species to clarify whether it is the larval or the adult diet that is more critical to reproductive development. It would also be interesting to investigate how dietary quality affects other aspects of mosquito reproductive physiology, such as the number of times that males mate with females, female receptivity to remating, the size of the blood meal taken by a female, and the number of eggs that a female lays over her lifetime. Taken together, our results improve our understanding of how nutrition affects the reproductive physiology of these deadly disease vectors, which could be useful in rearing more fit individuals that are meant for use in projects involving methods such as the sterile insect technique (Benedict and Robinson 2003), where the survival of released insects is critical. More broadly, these results may be helpful in

improving the fecundity of lab-reared mosquitoes as well as other insects of conservation importance.

5. References

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